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Leucine Zipper Dimerized Bivalent and Bispecific scFv Antibodies from a Semi-synthetic Antibody Phage Display Library*

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This report describes the construction of leucine zipper-based dimerization cassettes for the conversion of recombinant monomeric scFv antibody fragments to bivalent and bispecific dimers. A truncated murine IgG3 hinge region and a Fos or Jun leucine zipper were cloned into four scFv fragments previously isolated from a synthetic antibody phage display library. Cysteine residues flanking the zipper region were introduced to covalently link dimerized scFv fragments. The secreted fusion proteins were shown to spontaneously and efficiently form stable Fos·Fos or Jun·Jun homodimers in the *Escherichia coli* periplasm at levels comparable to their monovalent counterparts. The bivalent $(scFv)_2$ fragments performed well in enzyme-linked immunosorbent assay, flowcytometric, and immunohistochemical analysis. Fos and Jun homodimer $(scFv)_2$ antibodies with different specificities could be reduced, reshuffled, and reoxidized to form preparations of functional bispecific $(scFv)_2$ Fos·Jun heterodimers. These Fos and Jun fusion protein cassettes provide a universal basis for the construction of dimeric scFv antibodies with enhanced avidity or dual specificity.

Surface display of Fab or single chain Fv (scFv) antibody fragments on filamentous phage particles in combination with an array of versatile selection procedures has become a powerful approach to obtain recombinant molecules with desired specificities and binding properties from large libraries (reviewed by Winter *et al.* (1994) and Burton and Barbas (1994)). The Fab and scFv fragments thus obtained are monovalent, whereas in many *in vitro* and *in vivo* applications, multivalency of antibody molecules is a desirable property. In addition, linking two or more binding sites efficiently increases the functional avidity of antibody molecules or results in the construction of antibodies with dual specificities (Plückthun, 1992). Several approaches have been employed to generate genetically engineered, multimerized antibody fragments. Bivalent (and bispecific) $(scFv)_2$ and $(Fab)_2$ fragments have been successfully produced by association of two molecules through flexible linker polypeptides, chemical cross-linking, and dimerization domains (reviewed by Holliger and Winter (1993)). In the latter approach, introduction of amphipathic helices or leucine zippers was shown to mediate dimerization of scFv or Fab fragments *in vivo* (Pack and Plückthun, 1992; Pack *et al.*, 1993, 1995; Kostelny *et al.*, 1992). These efforts have resulted in the production of higher valency antibody fragments with widely varying physicochemical properties.

In designing strategies for dimerization of antibody fragments, several issues need to addressed including stability and homogeneity of the dimers, resistance to proteolytic cleavage during *in vivo* assembly, efficient production of preferably soluble protein, simple engineering steps, and general applicability for the construction of both bivalent and bispecific recombinant antibodies. With these issues in mind, we designed dimerization cassettes that allow the conversion of scFv antibodies from a number of published phage display libraries to bivalent or bispecific reagents involving a single cloning step. In this procedure, the flexible and proteolysis-resistant truncated mouse IgG3 upper hinge region (Pack and Plückthun, 1992) and either Fos or Jun leucine zippers were fused to scFv proteins. Two cysteine residues were engineered in the Fos and Jun zipper domains to produce disulfide-stabilized homodimers. Using four scFv antibodies previously isolated from a synthetic phage display library, we show that this approach results in the efficient *in vivo* production of stable, secreted homodimers that retain their specificity as assessed in a number of assays. Furthermore, exploiting preferential Fos·Jun heterodimer over Fos·Fos or Jun·Jun homodimer formation, we show that *in vitro* reduction, mixing, and re-oxidation of Fos and Jun scFv antibodies with different specificities results in the production of bispecific $(scFv)_2$ molecules.

MATERIALS AND METHODS

scFv Fragments—The scFv antibodies were selected from a semi-synthetic antibody phage display library constructed in the phagemid vector pHEN1. scFv clone 3 is specific for an IgG paraprotein, scFv clones 22 and 23 are specific for dinitrophenol coupled to bovine serum albumin (DNP-BSA),[1] scFv clone 35 is specific for the α chain of the CD8 molecule expressed on a subpopulation of human T lymphocytes, and scFv clone 40 is specific for the β chain of the CD22 molecule expressed on mature human B lymphocytes. All scFv molecules have been described in detail elsewhere under different names (clone 3, IgG2; clone 22, DNP2; clone 23, DNP5 (de Kruif *et al.* 1995a); clone 35, T1; clone 40, B28 (de Kruif *et al.* 1995b)).

Construction of scFv-Zipper Proteins—Oligonucleotide primers (Table I) were developed to append *Not*I restriction sites and a murine IgG3 upper hinge region (Pack and Plückthun, 1992) to modified Fos and Jun leucine zipper regions. At the N and C termini, the modified zipper regions contain cysteine residues added via Gly-Gly spacers (Crameri and Suter, 1993). Template consisting of the plasmid pJuFo (encoding both modified zipper genes; Crameri and Suter (1993)) was polymerase chain reaction amplified with the appropriate primer set (30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C for 25 cycles), digested with *Nod*, and cloned into phagemid vector pHEN1 (Hoogenboom *et al.*, 1991) to yield pHEN1-hFo and pHEN1-hJu. Sequences were verified by the dideoxy chain termination procedure. *Not*I digestion fragments from pHEN1-hFo and pHEN1-hJu were ligated into *Not*I-digested pHEN1-scFv clones to produce clones 3F (anti-IgG-hinge-Fos), 23J (an-

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[1] The abbreviations used are: DNP-BSA, dinitrophenol coupled to bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

TABLE I
Oligonucleotides used to construct hinge-leucine zipper regions
*Not*I sites used for cloning are underlined.

5'HIFO	5'-ATCAAACGGGCGGCCGCACCTAAACCCTCCACC-CCGCCTGGTTCTTCATGCGGTGGTCTGACC
3'HIFO	5'-TTTTTGTTCTGCGGCCGCGCAACCACCGTGTGC
5'HIJU	5'-ATCAAACGGGCGGCCGCACCTAAACCCTCCACC-CCGCCTGGTTCTTCATGCGGTGGTCGGATC
3'HIJU	5'-TTTTTGTTCTGCGGCCGCGCAACCACCGTGGTTC

ti-DNP-hinge-Jun), 35F (anti-CD8α-hinge-Fos), and 40J (anti-CD22β-hinge-Jun). Diagrams of the resulting fusion proteins are shown in Fig. 1.

Expression of scFv Proteins—scFv and $(scFv)_2$ proteins were expressed in *Escherichia coli* strain SF110 (Meerman and Georgiou, 1994), modified to contain the F' episome of *E. coli* XL1-Blue. Induction of protein synthesis and isolation of scFv fragments from the periplasmic space was performed as described (de Kruif *et al.*, 1995a).

SDS-PAGE and Western Blotting—Samples were separated on 10% SDS-polyacrylamide gels followed by electroblotting to nitrocellulose membranes. scFv proteins were visualized by staining with undiluted hybridoma supernatant containing the Myc tag-specific antibody 9E10 (9E10 SN), followed by a horseradish peroxidase-conjugated goat anti-mouse antibody (DAKO, Denmark) diluted 1/1000 in 4% milk powder-PBS (MPBS). In non-reducing SDS-PAGE, samples were preincubated in 60 mM iodoacetamide to block free sulfhydral groups before boiling in SDS-containing sample buffer.

Affinity Purification of Functional $(scFv)_2$ Molecules—Tosil-activated paramagnetic beads (Dynal, Norway) were coated overnight in 400 μg/ml IgG or DNP in borate buffer, pH 9.5. The beads were then blocked for 4 h in 2% MPBS. 200-μl $(scFv)_2$ periplasmic preparations were diluted 1/1 in 4% MPBS and added to the beads. After another 2-h incubation, the beads were washed five times in PBS, 0.1% Tween 20 (PBST), boiled in non-reducing sample SDS buffer, and subjected to SDS-PAGE analysis as described.

ELISA—Wells of microtiter plates (Nunc Maxisorp) were coated overnight at room temperature with DNP-BSA, IgG paraprotein, thyroglobulin, lysozyme, HMG-box protein, ovalbumin, or non-fat milk at 10 μg/ml in 50 mM $NaHCO_3$ (pH 9.6). Excess antigen was removed, after which the wells were blocked for 2 h in 2% MPBS. 100-μl scFv and $(scFv)_2$ preparations were diluted 1/1 in 4% MPBS and added to the wells. After a 2-h incubation, wells were washed in PBST, incubated in undiluted 9E10 SN, washed with PBST, and finally incubated with horseradish peroxidase-conjugated polyclonal goat-anti-mouse antibody (1/1750 in 2% MPBS). After a final wash in PBST, plates were developed using 2',2'-amino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium as a substrate.

For sandwich ELISAs, 50 μl of 3F × 23J (IgG × DNP) bispecific $(scFv)_2$ preparation was blocked in 150 μl of 4% MPBS for 15 min before addition to IgG-coated wells. After a 1-h incubation, plates were washed in PBST, and 200 μl of 1 μg/ml DNP-BSA in MPBS was added to the wells. Following another 1-h incubation, unbound DNP-BSA was removed by washing in PBST, and bound DNP-BSA was detected using clone 22 phage antibodies recognizing a different epitope on DNP than scFv clone 23. Binding of the clone 22 phage antibodies was visualized by incubation with a polyclonal sheep anti-M13 horseradish peroxidase-conjugated antibody (Pharmacia, Uppsala, Sweden) as described (de Kruif *et al.*, 1995a). All incubations were performed at room temperature.

Immunohistochemical Staining—COS7 cells were transfected with CD22β cDNA cloned into the CDM8 vector using DEAE-dextran sulfate and plated in Petri dishes according to standard protocols. 48 h after transfection, plates were washed in PBS, 1% BSA (PBSB) and incubated for 1 h with Jun-linked clone 40 $(scFv)_2$ antibodies diluted 1/2 in 4% MPBS. Plates were washed in PBSB and incubated with 9E10 SN for 1 h, followed by washing in PBSB and incubation in goat-anti-mouse antibodies coupled to horseradish peroxidase diluted 1/1000 in PBSB. Plates were developed in 3-amino-9-ethyl-carbazol and photographed. All incubations were performed at 4 °C.

Fluorescence-activated Cell Sorter Analysis—Peripheral blood leukocytes were isolated by Ficoll-Hypaque density centrifugation and stained with clone 35 scFv and Fos-linked clone 35 $(scFv)_2$ proteins using the anti-Myc monoclonal antibody and a phycoerythrin-conjugated goat anti-mouse IgG polyclonal antibody as second and third step reagents as described (de Kruif *et al.*, 1995b). Double staining was performed with a conventional fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibody (Becton Dickinson). In control staining experiments, incubations with scFv and $(scFv)_2$ fragments were omitted from the procedure.

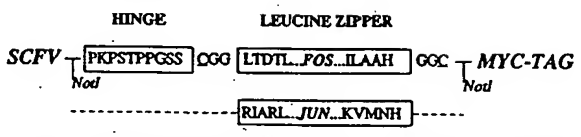

FIG. 1. **Diagram of the Fos and Jun dimerization cassettes cloned into scFv-containing pHEN1 phagemids.** Cysteine residues are *underlined*.

Formation of Anti-IgG × Anti-DNP Bispecific $(scFv)_2$ Fragments—Periplasmic preparations of clones 3F and 23J containing approximately equal amounts of $(scFv)_2$ homodimers were reduced in 32 mM 2-mercaptoethanol at 37 °C for 1 h, mixed, and dialyzed against redox buffer (50 mM Tris-HCL, pH 8.5, 1 mM EDTA, 500 μM reduced glutathione, and 500 μM oxidized glutathione) for 24 h at 4 °C. Subsequently, the buffer was changed back to PBS by dialysis.

RESULTS

Construction of scFv-Leucine Zipper Fusion Proteins—A polymerase chain reaction approach was used to append restriction sites and the mouse IgG3 upper hinge region to modified Fos and Jun leucine zippers. These "dimerization cassettes" were subsequently cloned into a unique *Not*I restriction site present in the 3'-end of the genes encoding scFv antibody fragments isolated from a semi-synthetic human phage antibody display library (Fig. 1).

SDS-PAGE and Western Blot Analysis—Equal volumes of periplasmic preparations containing expressed scFv fragments or their Fos and Jun fusion protein derivatives were run on an SDS-PAGE gel and analyzed by Western blotting using the anti-Myc tag antibody 9E10. Under reducing conditions, equal amounts of scFv fragments are detectable in the periplasm of scFv, scFv-hinge-Fos (3F), and scFv-hinge-Jun (23J) expressing bacteria, indicating that expression levels of the scFv molecules are not affected by addition of the hinge region and leucine zipper domains (Fig. 2). A shift in the gel mobility of the scFv-zipper proteins is observed, corresponding to the 56 amino acids introduced by the hinge region and zipper domains. When SDS-PAGE is performed under non-reducing conditions, a set of proteins, with closely spaced bands, corresponding to approximately twice the size of scFv-zipper proteins is detected solely in periplasmic preparations containing zipper constructs. No protein bands corresponding to the size of monomeric scFv zippers are present (Fig. 2).

To investigate the antigen binding potential of the closely spaced $(scFv)_2$ bands (Fig. 2), periplasmic preparations of Fos-dimerized anti-IgG (3F) and Jun-dimerized anti-DNP $(scFv)_2$ (23J) were allowed to bind to DNP-BSA and IgG coated to paramagnetic beads. After washing, bound proteins were eluted from the beads and analyzed by non-reducing SDS-PAGE. In control incubations, no binding of 23J $(scFv)_2$ to IgG or 3F $(scFv)_2$ to DNP was observed (Fig. 3, *lanes 2* and *6*). In contrast, immunoaffinity selection of 3F and 23J scFv dimers on the corresponding antigen-coated beads resulted in the purification of a set of proteins displaying the same characteristic banding pattern as their non-purified counterparts (Fig. 3, *lanes 1* and *3–5*). These results suggest that all $(scFv)_2$ proteins formed in the bacterial periplasm retained their specific antigen binding capacity.

Specificity of Bivalent $(scFv)_2$ Antibody Molecules in ELISA—ELISA assays were performed to assess the specificity of Fos- and Jun-linked bivalent $(scFv)_2$ molecules. *E. coli* SF110 cells were transformed with constructs encoding the 3F and 23J $(scFv)_2$ antibodies. Periplasmic preparations of cells containing approximately similar concentrations of antibody were incubated with DNP-BSA, IgG, and six control antigens coated to wells of a microtiter plate. Bivalent antibodies 3F and 23J

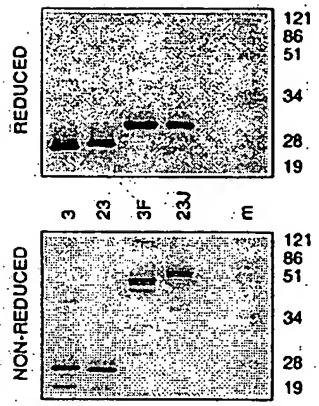

FIG. 2. **SDS-PAGE Western blot analysis of expressed scFv and $(scFv)_2$ proteins.** The *upper panel* shows the migration of anti-IgG (*lane 1*) and anti-DNP (*lane 2*) scFv and their Fos and Jun fusion protein derivatives (*lanes 3* and *4*, respectively) run under reducing conditions. In the *lower panel*, reducing agents were omitted from the sample SDS buffer.

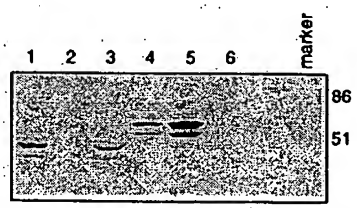

FIG. 3. **SDS-PAGE Western blot analysis of $(scFv)_2$ proteins selected for binding to their target antigen.** $(scFv)_2$ proteins were incubated with paramagnetic beads coated with either IgG or DNP. After washing, the beads were boiled in non-reducing SDS sample buffer, and the resulting protein mixture was applied to the gel. *Lane 1*, 3F $(scFv)_2$ periplasm; *lane 2*, 3F $(scFv)_2$ selected on DNP; *lane 3*, 3F $(scFv)_2$ selected on IgG; *lane 4*, 23J periplasm; *lane 5*, 23J $(scFv)_2$ selected on DNP; *lane 6*, 23J $(scFv)_2$ selected on IgG.

bound to IgG and DNP, respectively, whereas no significant binding to any of the control antigens was observed (Fig. 4).

Performance of the Anti-CD8α $(scFv)_2$ in Flow Cytometric Analysis—Peripheral blood mononuclear cells were incubated with periplasmic preparations of bacteria-producing $(scFv)_2$ antibodies against the CD8α chain expressed on a subpopulation of human T lymphocytes. Costaining with a fluorescein isothiocyanate-labeled CD8 monoclonal antibody shows that the $(scFv)_2$ molecules brightly and specifically stain the CD8 positive cells (Fig. 5). Note that some nonspecific staining of lymphocytes is caused by the second and third step antibodies.

Immunohistochemical Staining of CD22β Transfected COS Cells with Bivalent $(scFv)_2$ Antibodies—COS cells were transfected with the CD22β cDNA cloned into the CDM8 vector. A periplasmic preparation from *E. coli* SF110 transformed with Jun-linked anti-CD22β $(scFv)_2$ (40J) was used to stain transfected cells. Results show an intense staining of approximately 10% of the transfected cells (Fig. 6), corresponding to the transformation efficiency in this particular experiment. No staining was observed when non-relevant $(scFv)_2$ molecules with anti-DNP specificity were used to stain CD22β-transfected COS cells (results not shown).

Formation and Performance of Anti-IgG × Anti-DNP Bispecific $(scFv)_2$ Molecules—The formation of bispecific $(scFv)_2$ molecules was examined using anti-DNP and anti-IgG scFv clones 3F and 23J. Periplasmic preparations from bacteria transformed with these constructs were reduced in 2-mercaptoethanol. Upon this treatment, all detected proteins are present as

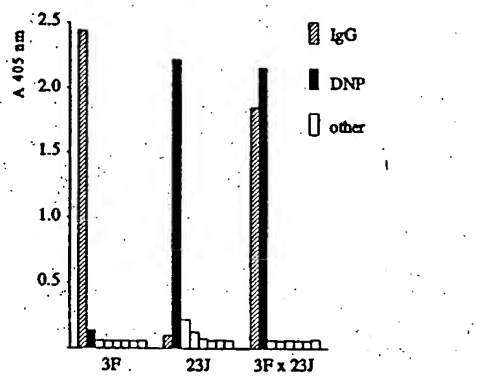

FIG. 4. **Antigen specificity of $(scFv)_2$ antibody fragments.** Microtiter plates were coated with IgG, DNP, or a panel of control antigens including lysozyme, thyroglobulin, ovalbumin, HMG-box protein, bovine serum albumin, and milk powder. $(scFv)_2$ molecules were allowed to bind and were detected using the 9E10 antibody.

scFv-zipper monomers (Fig. 7). To allow formation of heterodimeric $(scFv)_2$ molecules, the proteins were mixed and incubated in a redox buffer. After subsequent dialysis in PBS, a set of proteins corresponding to the size of $(scFv)_2$ molecules was observed (Fig. 7). Binding properties of these reshuffled $(scFv)_2$s was first examined in ELISA. The 3F × 23J protein preparation detects both the DNP and the IgG antigen (Fig. 4). No binding to other antigens is detected. To test the bispecific properties of the reshuffled proteins, a sandwich ELISA was performed. The proteins were allowed to bind to IgG-coated ELISA plates. After washing, DNP was added to the wells. Bound DNP was detected by an anti-DNP phage antibody followed by a horseradish peroxidase-conjugated anti-M13 antibody. A strong signal developed in the wells incubated with the 3Fx23J bispecific antibody (Fig. 8). No signal was observed in wells incubated with 3F or 23J homodimers or when DNP is omitted in the procedure.

DISCUSSION

We have constructed scFv antibody fragment dimerization cassettes that can be readily introduced in the *Not*I restriction sites of genes encoding scFvs isolated from a variety of phage display libraries described in the literature (Hoogenboom and Winter, 1992; Nissim *et al.*, 1994; de Kruif *et al.*, 1995a). These cassettes add a truncated, flexible murine IgG3 hinge region and either a Fos or Jun leucine zipper to the scFv proteins. To increase stability of the bivalent antibodies, cysteine residues were incorporated at the N and C termini of each of the leucine zippers, facilitating disulfide bridge formation in the periplasmic space (Crameri and Suter, 1993).

The performance of zipper-linked $(scFv)_2$ molecules was assessed using four different scFv antibodies selected from a synthetic phage display library as starting material. All scFv-zipper molecules were secreted as soluble proteins into the periplasmic space, obviating tedious refolding procedures associated with the formation of insoluble inclusion bodies (Kurucz *et al.*, 1995). In each instance, the level of expression did not appear to be significantly affected by addition of the hinge and Fos or Jun zipper regions. In Western blotting under non-denaturing conditions, periplasmic preparations of Fos or Jun scFv zippers solely contained dimeric molecules, indicating that the formation of $(scFv)_2$ homodimers from scFv-zipper monomeric precursors is extremely efficient. The homodimers were resistant to boiling in sample buffer containing 4% SDS and could only be dissociated to monomers using reducing agents. We conclude that the monomers in a $(scFv)_2$ complex are covalently linked via disulfide bridges connecting the

Previously, a tendency of GCN4 zipper-linked "mini-antibodies" to display nonspecific binding to antigens coated to microtiter wells has been noted (Pack *et al.* 1993). We examined the binding specificities of our bivalent and bispecific $(scFv)_2$ fragments in a number of assays, including ELISA, flow cytometry and immunohistochemistry. In none of these assays, significant nonspecific binding was observed. A reason for this apparent discrepancy between GCN4 zippers and Fos/Jun zippers may be a better shielding of the hydrophobic regions in the latter and/or the more stable configuration caused by covalently cross-linking the zipper regions.

Employing the much greater tendency of Fos and Jun zipper peptides to form heterodimers over homodimers (O'Shea *et al.*, 1989; Kostelny *et al.*, 1992), bivalent Fos and Jun leucine-zippered $(scFv)_2$ can be rapidly converted to bispecific $(scFv)_2$ molecules by simple reduction, mixing, and reoxidation steps. Using this approach, the anti-IgG and anti-DNP binding activities of two $(scFv)_2$ homodimers were shown to be combined in a single heterodimeric molecule. A major advantage of this strategy is that only a single straightforward cloning step is required to produce bispecific antibodies obviating the need for extensive polymerase chain reaction and cloning efforts (Holliger *et al.*, 1993; Mallender and Voss, 1994; Kurucz *et al.*, 1995; Mack *et al.*, 1995).

The dimerization system described here may be used to construct phage display libraries of bispecific antibodies. Bispecific antibodies that simultaneously recognize adjacent and non-overlapping epitopes on a target protein have higher avidities than the single chain or Fab antibodies obtained from conventional libraries (Neri *et al.*, 1995). Thus, a Fos-linked scFv with a desirable specificity may be cloned into a phage library of Jun-scFv antibodies, permitting the direct recovery of high avidity bispecific antibodies using stringent selection procedures. We are currently performing experiments to assess the feasibility of this approach.

We show that using cysteine-modified Fos and Jun leucine zipper peptides, scFv antibody fragments isolated from phage display libraries can be simply converted to functional bivalent and bispecific molecules involving only a single cloning step. It is important to note that scFv molecules obtained from phage display libraries have been through a stringent selection for correct expression, transport, and folding in bacterial cells. This explains why these antibodies and the derivatives described in this paper do not appear to suffer from many of the problems associated with bacterially expressed scFvs derived from hybridomas.

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